Journal of Experimental Botany, Vol. 55, No. 400,
Understanding Photosynthetic Performance Special Issue, pp. 1221–1230, May 2004
DOI: 10.1093/jxb/erh143 Advance Access publication 7 May, 2004

Journal of Experimental Botany

Responses of primary and secondary metabolism to sugar accumulation revealed by microarray expression analysis of the *Arabidopsis* mutant, *pho3*

Julie C. Lloyd* and Oksana V. Zakhleniuk

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, UK

Received 26 November 2003; Accepted 4 March 2004

Abstract

The *Arabidopsis* mutant *pho3* accumulates sucrose and other carbohydrates to high levels, providing a means of investigating the genomic response to sucrose accumulation using microarray analysis. Wild-type and mutant plants were grown in soil to the mature rosette stage for the analysis of gene expression using the Affymetrix ATH1 chip, containing more than 22 500 probe sets. Small, but significant, decreases were observed in the expression of many genes encoding enzymes and regulatory proteins involved in primary carbon assimilation, suggesting that, in mature leaves of *Arabidopsis,* there is limited feedback regulation on gene expression by sugars. The study revealed a striking increase in the expression of the plastid glucose 6-phosphate/phosphate translocator, characteristically expressed only in heterotrophic tissues. This indicated a change in the nature of metabolite exchange between the plastid and the cytosol in the *pho3* mutant. The expression of enzymes of starch synthesis also increased significantly. Very large increases were observed in the expression of transcription factors and enzymes involved in anthocyanin biosynthesis. This finding reinforces the emerging picture of an important role for primary metabolism in regulating secondary metabolism.

Key words: Anthocyanin, *Arabidopsis thaliana*, GPT, MYB proteins, primary metabolism, *suc2*, sucrose, sugar signalling.

Introduction

A principal feature of plant metabolism is the flexibility to accommodate developmental changes and to respond to the environment, where there can be alterations in light levels, temperature, and nutrient availability. On a short time-scale, metabolic regulation can be achieved through mechanisms such as altered enzyme kinetics in response to metabolite concentrations or via secondary modifications of protein structure, including phosphorylation and redox regulation. On a longer time-scale, gene transcription has also been shown to have a role in regulating metabolism. A significant literature now exists on the signalling networks which allow plants to perceive and respond to their environment and to developmental changes. One regulatory signal to which a number of plant genes have been shown to respond is carbohydrate status and this field has been widely reviewed (Koch, 1996; Smeekens, 2000; Rolland *et al*., 2002). Genes encoding enzymes and other proteins involved in assimilation, storage, and mobilization of reserves have been found to respond at the transcriptional level to both sugar accumulation and depletion. For this reason it has been suggested that sugar signals may be important in regulating source–sink status, and carbon partitioning in the plant (Roitsch, 1999; Paul and Foyer, 2001). As yet, no detailed picture of the signalling mechanisms has emerged, but some potentially important components have been identified. These include a possible role for hexokinase and the links between sugar and hormone signals in seedling development (Finkelstein and Gibson, 2002; Leon and Sheen, 2003). Many of the recent developments in the field of sugar signalling have come from the identification of mutants with altered sugar responses and this approach should continue to provide

* To whom correspondence should be addressed. Fax: +44 1206 872592. E-mail: lloyj@essex.ac.uk

valuable insights into the signalling pathways (Smeekens, 2000).

Many of the experiments on sugar responses have focused on genes involved in primary metabolism. However, there is also a small amount of evidence from transgenic plants that fluxes through primary metabolic pathways can impact on the export of intermediates into secondary pathways. Reductions in the Calvin cycle/oxidative pentose phosphate pathway enzyme, transketolase, have been shown to impact on flux into shikimate and phenylpropanoid metabolism (Henkes *et al*., 2001). Elucidation of these regulatory networks might be important not only in terms of understanding the limitations on plant productivity, but also for the successful exploitation of plant metabolism to manipulate end, or novel, product accumulation.

One approach that allows the expression of many genes to be studied in a single experiment, without introducing bias by pre-selection, is microarray analysis. Microarray analysis has been used here to investigate the effect of sucrose accumulation on gene expression. The *pho3* mutant of *Arabidopsis* has recently been shown to harbour a defective copy of the *SUC2* gene (OV Zakhleniuk and JC Lloyd, unpublished data). The *SUC2* gene encodes a sucrose-proton symporter that is important for phloem loading of sucrose (Gottwald *et al*., 2000) and, as a consequence of this mutation, the *pho3* plants accumulate large amounts of both soluble sugars (glucose, fructose, and sucrose) and starch. The mutant also has severely reduced growth rates and a distinctive purple leaf colour as a result of increased anthocyanin pigmentation, particularly when grown in soil (Zakhleniuk *et al*., 2001). The sugar accumulation observed in the *pho3* mutant makes it potentially useful for investigating regulatory pathways that respond to the products of primary carbon assimilation. This paper describes the results of microarray analysis to investigate the effect of the *pho3* mutation on metabolic gene expression. The findings are discussed in the context of previous work on the function and expression of these genes.

Materials and methods

Plant growth

Wild-type *Arabidopsis* ecotype Wassilewskija and *pho3* mutant seeds were surface-sterilized and sown onto half-strength MS medium containing 1% Suc (w/v). After 10 d, seedlings were removed into compost (Fisons Levington) and transferred to a controlled environment cabinet (22 °C; 140 µmol m^{-1} s^{-1} light; 16 h day length). Plants were harvested once they had grown to the equivalent of developmental stage 6.00 which is when the first flower is open (Boyes *et al*., 2001). Tissue samples were harvested from 10–15 plants and snap frozen in liquid nitrogen. Material was stored at –80 °C prior to RNA extraction. Three independently grown, harvested, and extracted sets of samples were prepared as biological replicates. Comparisons of the data from these replicates and complementary expression analysis using northern blotting (data not shown) indicated that one control and one mutant sample were not giving robust representative results. The data presented here are, therefore, the average of two biological replicates for wild-type and *pho3* plants.

RNA extraction

RNA was extracted using TRI REAGENT™ (Sigma) according to the protocol supplied by the manufacturer, with modifications which included double chloroform wash and two additional precipitations of RNA with ethanol. Samples of total RNA were sent to the Nottingham *Arabidopsis* Stock Centre for probe preparation and *Arabidopsis* GeneChip analysis (Affymetrix Inc., Santa Clara, CA). RNA yield and purity were determined spectrophotometrically, and their integrity was checked at the Nottingham *Arabidopsis* Stock Centre (NASC) using an Agilent 2100 Bioanalyser (Agilent Technologies, Boblingen, Germany).

Approximately 5 µg of total RNA was reverse transcribed at 42 °C for 1 h to generate first-strand DNA using 100 pmol oligo dT(24) primer containing a 5′-T7 RNA polymerase promoter sequence, 50 mM TRIS-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 10 mM dNTPs, and 200 units SuperScript II reverse transcriptase (Invitrogen Life Technologies). Following first-strand synthesis, second-strand DNA synthesis was synthesized using 10 units of *E. coli* polymerase I, 10 units of *E. coli* DNA ligase, and 2 units of RNase H in a reaction containing 25 mM TRIS-HCl (pH 7.5), 100 mM KCL, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM b-NAD^+, and 10 mM dNTPs. The second strand synthesis reaction proceeded at 16 °C for 2 h before 10 units of T4 DNA polymerase was added and the reaction allowed to proceed for a further 5 min. The reaction was terminated by adding 0.5 M EDTA. Double-stranded cDNA products were purified using the GeneChip Sample Cleanup Module (Affymetrix).

The synthesized cDNAs were *in vitro*-transcribed by T7 RNA polymerase (ENZO BioArray High Yield RNA Transcript Labeling Kit, Enzo) using biotinylated nucleotides to generated biotinylated complementary RNAs (cRNAs). The cRNAs were purified using the GeneChip Sample Cleanup Module (Affymetrix). The cRNAs were then randomly fragmented at 94 °C for 35 min in a buffer containing 40 mM TRIS-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate to generate molecules of approximately 35–200 bases long.

The Affymetrix ATH1 *Arabidopsis* genome GeneChip array was hybridized with 15 µg of the fragmented labelled cRNA for 16 h at 45 °C as described in the Affymetrix Technical Analysis Manual. GeneChips were stained with Streptavidin-Phycoerythrin solution and scanned with an Agilent G2500A GeneArray Scanner (Agilent Technologies). Microsoft Excel (Microsoft, Redmont, WA) was used to manage and filter the microarray data using MAS 5.0 (Affymetrix) signals. Functional categories were assigned to genes using the AGI number to search the MIPS database (http://mips.gsf.de/cgi-bin/proj/thal/) and the *Arabidopsis* Information Resource website, TAIR (http://www.arabidopsis.org/). The full set of microarray data from this experiment are available on public access at the Nottingham *Arabidopsis* Stock Centre website (http://nasc.nott.ac.uk/).

Results and discussion

To investigate the effect of the *pho3* mutation on genome-wide metabolic gene expression a comparison was made of transcript levels in mature rosette leaves of wild-type and *pho3* mutant *Arabidopsis* plants. RNA samples (two biological replicates of each, from independent experiments) were analysed using the Affymetrix GeneChip

Arabidopsis array, Ath1, representing over 22 000 genes. The output from the Affymetrix Microarray Suite software analysis assigns probe sets giving a reliable expression signal as 'present'. Using this information a total of 10 922 genes were found to be expressed in both wild-type replicates and a larger number of genes, 12 485, were expressed in the *pho3* mutant samples. Data from the array were averaged for the two replicates and a value for the signal intensity (RNA expression level) obtained. An overall view of the differences in gene expression between the *pho3* mutant and wild-type plants was obtained by comparing the average signal intensities for individual genes in the two samples (Fig. 1). The trend observed in the *pho3* mutant was for increased levels of expression of individual genes and, in particular, to switching on the expression of many genes that were not expressed in the wild-type plant.

This response was confirmed when comparisons were made of the numbers of genes showing increased or decreased expression in the *pho3* mutant compared with wild-type plants (Table 1). Two-fold changes are generally regarded as indicating a significant difference in expression. However, in the *pho3* mutant many genes showed much larger changes in expression, so the numbers of genes with 3- and 5-fold changes were also calculated. The trend observed was always for more genes with increased expression in the *pho3* plants (Table 1), confirming the visual impression from the scatter plot (Fig. 1).

The fold-change in signal is one indication of a significant change in expression, but more weight can be placed on these data if the expression signal is high. It was found that 211 of the genes with at least 5-fold increased expression had an average signal of 100 or more. This compared with only 48 of the genes with at least a 5-fold decrease in expression. To allow the nature of the changes in gene expression taking place in the mutant to be unravelled, those genes showing the greatest changes in expression were assigned to a number of functional categories (Fig. 2).

The largest group of genes are those with unknown function, and not enough similarity to any other genes to assign a likely role in the plant on the basis of sequence homology. Genes involved in signal transduction, defence or stress-related functions, and secondary metabolism were notably more highly represented among the group of genes with increased expression. By contrast, five genes involved in development or homone-related processes had very large decreases in expression in the *pho3* mutant, but, interestingly, none in this category was increased. In order to analyse the changes in expression in more detail, the data for specific groups of genes were considered.

Sucrose transporter genes

Nine members of the sucrose transporter gene family (sucrose symporters, named SUC or SUT) have been

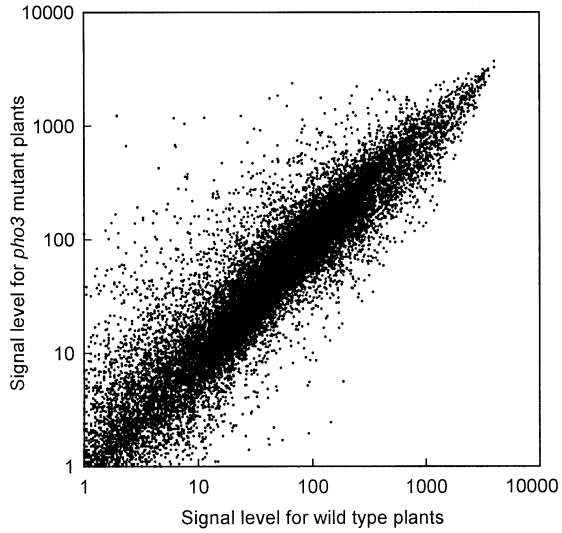

Fig. 1. Scatter plot comparison of the *pho3* mutant and wild-type *Arabidopsis* expression profiles. The normalized expression value for each gene probe set in the *pho3* mutant plants is plotted on the vertical axis and the equivalent value for wild-type plants on the horizontal axis. Both axes are logarithmic.

Table 1. *Numbers of genes showing altered expression in the* pho3 *mutant*

Genes showing a change in expression of at least	Number increased in *pho3* mutant	Number decreased in *pho3* mutant
2-fold	1305	844
3-fold	613	230
5-fold	320	56

identified in *Arabidopsis* (Lalonde *et al*., 1999). Recently, nucleotide sequence data were obtained to show that the *pho3* mutation was a single base splice-site mutation in the *SUC2* gene. This result was in keeping with northern blot analysis which indicated lower levels of an aberrant sized transcript in the *pho3* mutant (OV Zakhleniuk and JC Lloyd, unpublished data). Microarray analysis confirmed this decrease in the SUC2 transcript level in the *pho3* mutant, to about 50% of the wild-type level (Table 2). Expression analysis in *Arabidopsis* has shown that AtSUC2 is expressed only in the companion cells of phloem, in both source and sink tissues (Truernit and Sauer, 1995; Stadler and Sauer, 1996). There is one report of sucrose regulation of the sugar beet sucrose symporter (SUT1) (Vaughn *et al*., 2002), however, in potato and tomato SUT1 expression was not affected by sucrose (Barker *et al*., 2000).

By contrast with AtSUC2, levels of AtSUC1, AtSUC3, and AtSUT4 transcripts were unchanged in the *pho3* mutant (Table 2). It is not clear why such a high signal was obtained for AtSUC1, since expression of this gene has

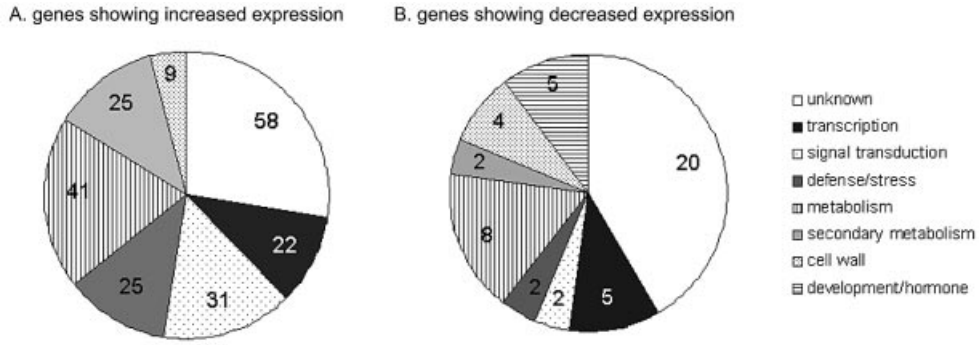

Fig. 2. Distribution of genes showing a 5-fold or greater change in expression in the *pho3* mutant into functional categories.

been reported to be specific to flowers (Stadler *et al*., 1999). The remaining five SUC1-like genes that make up this family in *Arabidopsis* did not give a detectable transcript signal on the microarray, indicating that they are not expressed at detectable levels in mature leaf tissue of wil-type *Arabidopsis*, and are also not induced in the *pho3* mutant.

Regulation of sugar movement into the vacuole for storage is likely to be an important feature of the plant response to excess sucrose, enabling the plant to avoid osmotic stress that might result from accumulation of this solute in the cytosol. As yet, no tonoplast sucrose transporter has been identified (Maeshima, 2001). Regulation of sugar movement across this membrane by transcriptional or post-transcriptional mechanisms could also be involved in metabolic signalling. It is interesting that, although very large changes in sugar levels occur as a result of the *pho3* mutation, no change was observed in the expression of any of the sucrose transporters identified in the *Arabidopsis* genome to date, with the exception of SUC2. The decrease in signals for this gene in the microarray experiment is likely to result from it being the site of the *pho3* mutation, rather than feedback due to sugar accumulation.

Plastid carbon metabolism

The transcript levels of genes encoding enzymes of primary carbon assimilation, the Calvin cycle, were reduced generally in the *pho3* mutant compared with levels found in the wild-type plants (Table 2). The small subunit of ribulose-1,5-*bis*phosphate carboxylase oxygenase (Rubisco) is encoded by a multigene family, of which only one member is represented on the microarray, and this highly expressed transcript decreased by less than 20% in the *pho3* mutant. Most other enzymes of the Calvin cycle were more convincingly reduced, to nearer 50% of their level in wild-type plants, for example, phosphoribulokinase and sedoheptulose-1,7-*bis*phosphatase. Other proteins involved in the catalytic regulation of primary carbon assimilation, Rubisco activase and thioredoxin f, had similar reductions in transcript levels of 40–60% (Table 2).

It is perhaps surprising that the Rubisco small subunit 1a transcript level changed so little in the *pho3* mutant, much less than the generally accepted 2-fold significance threshold for changes in a microarray experiment. In mature plants, feeding of sugar to detached leaves or increasing the sugar levels within individual leaves by inhibiting transport out of the leaf, have been reported to lead to reductions in transcript levels for the small subunit of Rubisco and other proteins involved in photosynthetic carbon assimilation (Krapp *et al*., 1991, 1993; Jang and Sheen, 1994; Krapp and Stitt, 1995; Jones *et al*., 1996). However, tobacco seedlings grown in agar with sugar had reduced Rubisco transcript levels only if they were also deficient in nitrogen (Nielsen *et al*., 1998). There are other examples of an absence of correlation between sugar accumulation and down-regulation of photosynthetic genes, for example, when flux into starch synthesis was reduced by antisense inhibition of ADP-glucose pyrophosphorylase in potato and also in the starchless mutants of *Arabidopsis* (Ludewig *et al*., 1998; Sun *et al*., 1999). These data, confirmed by the present microarray analysis, clearly show that feedback regulation of photosynthetic gene expression is not a major feature of the response of plants to long-term accumulation of end-products. The implication of this is that plants have other mechanisms which enable them to store the photosynthate or divert it into other pathways, rather than shutting down carbon assimilation.

By contrast with the relatively small changes in Calvin cycle transcripts, some much larger changes were observed for genes of starch synthesis (Table 2). The enzyme ADP-glucose pyrophosphorylase (AGPase) catalyses a regulatory step in starch biosynthesis. AGPase contains two subunits, referred to as the small and large subunits, and substantial increases in the expression of two AGPase large subunit genes (AtAPL3 and AtAPL4) were detected in the *pho3* mutant. The AtAPL3 transcript increased by 7-fold and an even greater change, 29-fold, was observed for the AtAPL4 transcript. The gene encoding the AtAPL3 subunit has previously been shown to be regulated by sucrose in a pathway involving abscisic acid (Rook *et al*.,

Table 2. *Expression of selected genes in rosette leaves of wild type and* pho3 *mutant* Arabidopsis *plants*

Affymetrix signal intensities for two biological replicates are given. The fold change in expression was calculated as the ratio of the average signals for *pho3* samples relative to the wild type. Genechip IDs are the Affymetrix probe set numbers.

ID	Gene number	Gene product	*pho3* signal		Wild-type signal		Fold-change
			1	**2**	**1**	**2**	
	Sucrose transport						
415243	At1g71880	SUC1 sucrose transporter	406	474	432	210	1.37
419304	At1g22710	SUC2	273	295	595	748	0.42
421882	At2g02860	SUC3/SUT2	110	97	73	72	1.43
419762	At1g09960	SUT4	44	46	46	45	0.98
	Chloroplast carbon metabolism						
419575	At1g67090	Rubisco small subunit 1a	3215	2899	3397	3715	0.86
406981	At3g54050	Fructose-1,6-*bis*phosphatase	803	731	1576	1602	0.48
405334	At5g13420	Transaldolase	298	270	351	311	0.86
406497	At3g60750	Transketolase	1461	1317	2071	2133	0.66
406862	At3g55800	Sedoheptulose-1,7-*bis*phosphatase	955	1110	2357	1991	0.48
410820	At1g32060	Phosphoribulokinase	1394	1478	2726	2805	0.51
417478	At1g73110	Rubisco activase	87	66	191	170	0.42
413707	At3g02730	Thioredoxin f1	805	960	1310	1352	0.66
407989	At4g39210	ADP-glucose pyrophosphorylase APL3	541	550	99	56	7
418645	At2g21590	ADP-glucose pyrophosphorylase APL4	458	488	16	16	29
	Plastid carbon transport						
401608	At5g16150	pGlcT plastidic glucose transporter	653	550	759	948	0.7
403986	At5g46110	TPT triose phosphate translocator	2354	2191	3230	3030	0.73
414285	At3g01550	PPT2 phosphoenolpyruvate translocator	42	54	220	153	0.26
401545	At5g17630	XPT pentose P translocator	115	119	250	249	0.47
403244	At5g54800	GPT1 glucose-6-P translocator	165	174	208	209	0.81
419500	At1g61800	GPT2 glucose-6-P translocator	1816	1862	57	42	37
	Intermediary metabolism						
415937	At1g43670	Fructose-1,6-*bis*phosphatase	340	355	842	786	0.43
405174	At5g17310	UDP-glucose pyrophosphorylase	111	164	256	205	0.6
413949	At3g03250	UDP-glucose pyrophosphorylase	993	983	374	391	2.58
401176	At5g20280	Sucrose phosphate synthase	907	913	366	422	2.31
401098	At5g20830	Sucrose synthase 1 SUS1	257	177	211	171	1.13
402438	At5g63680	Pyruvate kinase	288	313	114	93	2.9
421790	At2g19900	Malate oxidoreductase (malic enzyme)	120	113	46	20	3.52
402160	At5g66760	Succinate dehydrogenase	580	567	200	160	3.19
	Anthocyanin biosynthesis						
418945	At2g37040	Phenylalanine ammonia-lyase PAL1	232	380	123	83	2.98
405308	At5g13930	Chalcone synthase CHS	2177	2548	98	36	35
405894	At5g05270	Chalcone isomerase CHI	157	256	7	4	37
407224	At3g51240	Flavonone hydroxylase F3H	662	1171	41	29	26
405659	At5g07990	Flavonoid 3′ hydroxylase F3′H	160	284	51	41	4.8
404315	At5g42800	Dihydroflavonol 4-reductase DFR	736	1155	34	28	31
409383	At4g22870	Leucoanthocyanidin dioxygenase LDOX	939	1403	9	3	190
	Regulation of anthocyanin biosynthesis						
400728	At1g56650	MYB transcription factor PAP1	343	625	18	50	9.6
415240	At1g66390	MYB transcription factor PAP2	535	800	2	2	284
410156	At4g09820	TT8 (basic helix loop helix protein)	22	48	1	1	47

2001). It would appear that the increase in starch levels in the *pho3* mutant is, at least in part, due to transcriptional control of starch biosynthesis genes. However, this enzyme is also known to be subject to allosteric control, ensuring that the Calvin cycle does not become depleted of intermediates for starch synthesis in preference to export for sucrose biosynthesis in the cytoplasm. Interestingly, recent work has indicated that post-transcriptional regulation of AGPase is responsive to sucrose (Hendriks *et al*., 2003), complementing the sugar regulation of AGPase gene expression to provide an additional short-term level of control to balance starch and sucrose synthesis. Starch mobilization from leaves is rather less well understood at present (Smith *et al*., 2003).

Work carried out in tobacco has suggested that phosphorus nutrition might also be important in regulating AGPase gene expression. Transcript levels of the regulatory small subunit of AGPase, encoded by the *AgpS2* gene, increased when seedlings were sugar fed, but only in plants that were also grown under phosphate deficiency (Nielson

et al., 1998). Interestingly, in the same paper repression of AGPase expression by sucrose feeding of detached mature leaves was also antagonized by phosphate.

The connection between phosphorus nutrition and sugar in regulating gene expression has been observed for a number of other genes involved in carbon metabolism, such as UDP-glucose pyrophosphorylase (Ciereszko *et al*., 2001). The soil-grown *pho3 Arabidopsis* plants used in this microarray experiment were supplied with nutrients and were not deficient in phosphate (OV Zakhleniuk and JC Lloyd, unpublished data). However, a small number of genes involved in phosphorus nutrition were increased in expression, including 5-fold changes in two phosphate transporters Pht1;4 and Pht1;5 (At2g38940 and At2g32830; named according to Mudge *et al*., 2002). It is possible that this effect on gene expression reflects not a general deficiency in phosphorus, but more subtle changes in particular phosphate pools. Such changes in phosphate, whilst challenging to detect, might contribute to the regulation of AGPase gene expression by sucrose in the *pho3* mutant.

Plastid phosphate translocators

The plastidic phosphate translocators, which share inorganic phosphate as a substrate, but exchange this for different specific sugar phosphates, have a central and crucial role in linking plastid and cytosolic carbon metabolism. In recent years, the molecular cloning and functional characterization of these transporters, together with information on their role *in vivo* from mutants and antisense plants with reduced or absent expression, has allowed a comprehensive picture to emerge of this area (reviewed by Knappe *et al*., 2003; Flugge *et al*., 2003). Most of the carbon fixed by mature leaves is exported from the chloroplast by the triose phosphate/phosphate translocator (TPT) for subsequent utilization in sucrose and amino acid biosynthesis in the cytoplasm. A reduction in the availability of cytosolic phosphate for triose phosphate exchange redirects carbon within the plastid into starch. At night, sucrose biosynthesis is supplied with carbon by export from the plastid of the hexose products of starch breakdown, via the hexose transporter, although at present the regulation of starch breakdown in leaves is only partly understood. The *Arabidopsis* genome contains only one TPT gene, almost exclusively expressed in photosynthetically active tissues. In the *pho3* mutant, expression of the TPT was essentially unchanged (Table 2). In marked contrast, the mutant showed a striking increase in the glucose 6-phosphate/phosphate translocator (GPT) to as much as 37-fold greater than found in the wild-type leaf (Table 2). The GPT has been cloned from storage tissues of a number of plant species where it is the main route for the supply of carbon into the plastid for starch biosynthesis (Kammerer *et al*., 1998). This gene is not normally expressed in photosynthetic tissues and the very large increase observed in the expression of this gene suggests that, in the *pho3* mutant, imported glucose 6-P is used as a substrate for starch synthesis. This source of carbon would normally be used only in heterotrophic storage tissues, but in the *pho3* mutant would appear to complement starch biosynthesis from intermediates supplied directly from the Calvin cycle.

The observed increase in expression of the GPT might also provide an important alternative mechanism for balancing the chloroplastic and cytosolic phosphate pools, since phosphate would be released from the imported hexose phosphate during starch synthesis. This is the first report of metabolic regulation of the GPT and it will be of interest to investigate whether the metabolic signals leading to the increase in GPT expression can be linked, directly or indirectly, to the accumulation of sucrose or another metabolite in the cytosol. Another translocator, the PPT2 that transports phosphoenolpyruvate into the plastid for shikimate synthesis, is significantly reduced in the *pho3* mutant. However, this may not limit the supply of PEP significantly since the *pho3* mutant is still able to synthesize large amounts of anthocyanins, which are products of phenylpropanoid biosynthesis and, therefore, depend on flux through the shikimate pathway.

Intermediary metabolism

Large changes in gene expression were not generally evident amongst genes for cytosolic carbon metabolism, however, where changes were observed, the trend was towards increased expression (Table 2 and below). The analysis was made more challenging since some of these enzymes are encoded by gene families, not all of which have been characterized in terms of their relative contribution or possible differential expression or compartmentalization. Some genes involved in sucrose synthesis increased slightly, including UDP-glucose pyrophosphorylase (2.5-fold increase) and sucrose phosphate synthase (gene expression increased 2-fold). However, transcript levels for a key enzyme in regulating flux to sucrose biosynthesis, cytosolic FBPase, decreased more than 2-fold, as did another gene encoding UDP-glucose pyrophosphorylase. Generally, sucrose accumulation does not appear to lead to widespread decreases in the expression of sucrose synthesis genes. Levels of the sucrose breakdown enzyme, sucrose synthase 1 (SUS1) transcript did not change. This observation differs from previous reports showing increased expression of this gene in response to exogenous sucrose and other sugars (Ciereszko and Kleczkowski, 2002; Baud *et al*., 2004). Osmotic stress also resulted in upregulation of SUS1 gene expression in these studies. It is surprising that neither of these signals appears to result in increased SUS1 gene expression in the *pho3* mutant and suggests the presence of distinct sugar signalling pathways for subsets of sugar

responsive genes. None of the other five members of the sucrose synthase gene family were expressed in wild-type or *pho3* mutant rosette leaves.

Other changes in expression included certain enzymes of glycolysis and the oxidative pentose phosphate pathway, but the changes were not consistent for every enzyme in the pathway. Pyruvate kinase expression increased 3-fold and more than one cytosolic glucose-6-phosphate dehydrogenase gene had increased expression levels, while, by contrast, plastid forms of this enzyme had unchanged, or reduced, transcript levels. Similarly, small upward trends were seen for transcript levels of some genes classified as involved in amino acid metabolism.

Expression of the mitochondrial tricarboxylic acid cycle genes also showed little change and those with significant differences in the *pho3* mutant were increased. Succinate dehydrogenase transcript levels increased 3-fold, but others, such as malic enzyme, revealed no evidence of altered expression in the *pho3* mutant.

Secondary metabolism

One notable aspect of the *pho3* mutant phenotype is the accumulation of anthocyanin pigments. Anthocyanins are secondary metabolites produced by the flavonoid biosynthetic pathway and are found in increased amounts in response to stress, including light and nutrient deficiency (reviewed by Winkel-Shirley, 2002). They are synthesized in the cytoplasm from phenylpropanoid precursors and are then transported to, and stored in, the vacuole. Many of the largest changes in expression observed in the *pho3* mutant were of genes involved in anthocyanin biosynthesis, and increases of between 5-fold and 190-fold were observed in the microarray gene expression data (Table 2). The increases were evident for enzymes catalysing sequential steps in the pathway from phenylalanine to anthocyanins (the enzymes PAL, CHS, CHI, F3H, F3′H, DFR, and LDOX; Table 2).

There is now considerable data showing that control of transcription is the major mechanism for regulating this pathway of secondary metabolism (Davies and Schwinn, 2003). The anthocyanin biosynthesis genes appear to be regulated as groups by the same transcription factors, several of which also showed striking increases in expression in the *pho3* mutant. Transcripts encoding two MYB proteins, PAP1 and PAP2, were expressed at very high levels in the *pho3* mutant (Table 2). The increases relative to wild type were also very large, with PAP2 expressed at almost 300-fold higher levels in leaves of *pho3* plants at the mature rosette stage. PAP1 was first identified as a result of the analysis of a dominant over-expression mutant with constitutive anthocyanin production (Borevitz *et al*., 2000). A similar mutation in a MYB transcription factor gene has recently been identified in tomato, leading not only to over-expression of anthocyanin biosynthesis genes but also to upregulation of genes involved in glycosylation and transport of these pigments (Mathews *et al*., 2003). Another transcription factor, the basic helix-loop-helix protein encoded by the *TT8* gene, also showed increased expression in the *pho3* mutant. This protein has been implicated in the expression of two of the enzymes responsible for the later stages of anthocyanin biosynthesis, DFR and BAN (anthocyanidin reductase) (Nesi *et al*., 2000). Although DFR expression increased in the *pho3* mutant, BAN expression did not as it requires another MYB transcription factor, encoded by the *TT2* gene and this is not expressed in leaves. The polymeric proanthocyanidins synthesized by BAN are confined to the seed coat and TT2 has been implicated in determining this tissue specific expression (Nesi *et al*., 2001).

An important step in anthocyanin accumulation is their transport to the vacuole, since these compounds are toxic and require an acidic pH for stability. However, the only anthocyanin transporter to have been identified is a toxic compound extrusion (MATE) transporter required for flavonoid accumulation in the seed coat vacuole (Debeaujon *et al*., 2001). It has been suggested that glutathione *S*-transferase acts as an anthocyanin binding protein, preventing anthocyanin oxidation or crosslinking in the cytosol and indirectly enhancing transport to the vacuole (Mueller *et al*., 2000; Edwards *et al*., 2000). In the *pho3* mutant several glutathione *S*-transferase genes show increased expression, including one with a 136-fold higher transcript level (At5g17220). Recently, this gene was identified as encoding a glutathione *S*-transferase essential for anthocyanin accumulation in leaves by analysis of the *tt19* mutant (Kitamura *et al*., 2004). These data support a functional role for this member of the large glutathione *S*-transferase gene family in anthocyanin sequestration in leaves.

Although the accumulation of anthocyanins is linked with responses to a number of different stresses, including nutrient limitation, pathogen attack, and excess light, there is no obvious mechanistic link between these and the accumulation observed in the *pho3* mutant. However, a possible role for the increased synthesis of anthocyanins in the *pho3* mutant could be to provide a sink for the excess carbon accumulating in the leaves. The discovery of the possible response of PAP1 and PAP2 expression to increased sugar levels provides a new tool for elucidation of signalling steps upstream of these transcription factors.

Sugar signalling

Sugars are known to perform important regulatory functions at all stages of the plant life cycle and it seems likely that a number of different signalling pathways respond to these metabolites. A picture of how some components of these sugar signalling pathways might function is beginning to emerge (reviewed in Smeekens, 2000; Halford and Paul, 2003). The data from the *pho3* microarray were studied to look for changes in transcript levels encoding

proteins which have been linked to these pathways. Work in transgenic plants has suggested that hexokinase might be involved in feedback regulation of photosynthetic gene expression (Jang *et al*., 1997; Moore *et al*., 2003). However, there is still a lack of evidence linking any particular plant hexokinase with this function and it has been observed that separating a potential role for hexokinase in sugar sensing from the catalytic function of the enzyme will prove to be challenging (Halford *et al*., 1999). No changes in hexokinase transcript levels were found in the *pho3* mutant, however, this does not rule out a signalling function for these proteins in the mutant.

The role of hexokinases in sugar sensing was first elucidated in yeast, where another protein complex, a protein-serine/threonine kinase, is involved in sugar signalling. This SNF1 kinase complex relieves glucose repression of gene expression in yeast when levels of this sugar are low. It is conserved in other species, including plants, where the components are encoded by multigene families (Halford and Hardie, 1998). Antisense experiments have indicated a role for a SNF1 homologue in sucrose induction of gene expression in plants (Purcell *et al*., 1998), but again there was no change in the SNF1 homologues transcript levels (AKIN10 and AKIN11) in the *pho3* mutant. Another protein which interacts with the *Arabidopsis* SNF1 homologues in a glucose-dependent manner, the WD40 repeat containing protein, PRL1, has recently been identified (Nemeth *et al*., 1998; Bhalerao *et al*., 1999). A knockout mutant in this gene has a pleiotropic phenotype and the function of PRL1 *in vivo* is not well understood. In the *pho3* mutant expression of PRL1 increased slightly, but less than 2-fold. However, the complex is not thought to be regulated at the level of transcription so this does not argue against a role in signalling. Interestingly, a putative *Arabidopsis* SNF3 kinase homologue increased by 4.6-fold in the *pho3* mutant.

A possible role for the direct sugar regulation of genes encoding components of sugar signalling pathways has recently emerged. A basic leucine zipper transcription factor, ATB2, was identified and expression of this protein was shown to be repressed by sucrose at the translational level. The regulatory mechanism appears to involve the recognition of external, or exogenously supplied sucrose, rather than that synthesized in the cytoplasm of the cell (Rook *et al*., 1998). Interestingly, in the *pho3* mutant the transcript levels of this gene were increased by more than 4-fold suggesting that its expression may be regulated by sugars at the level of transcription, in addition to translation.

Recently, interest has grown in the possible role of trehalose-6-phosphate in regulating sugar metabolism, particularly in source/sink regulation and signalling (Eastmond *et al*., 2003). The *pho3* microarray data were used to investigate the expression of 22 genes identified as potentially involved in trehalose metabolism by Eastmond

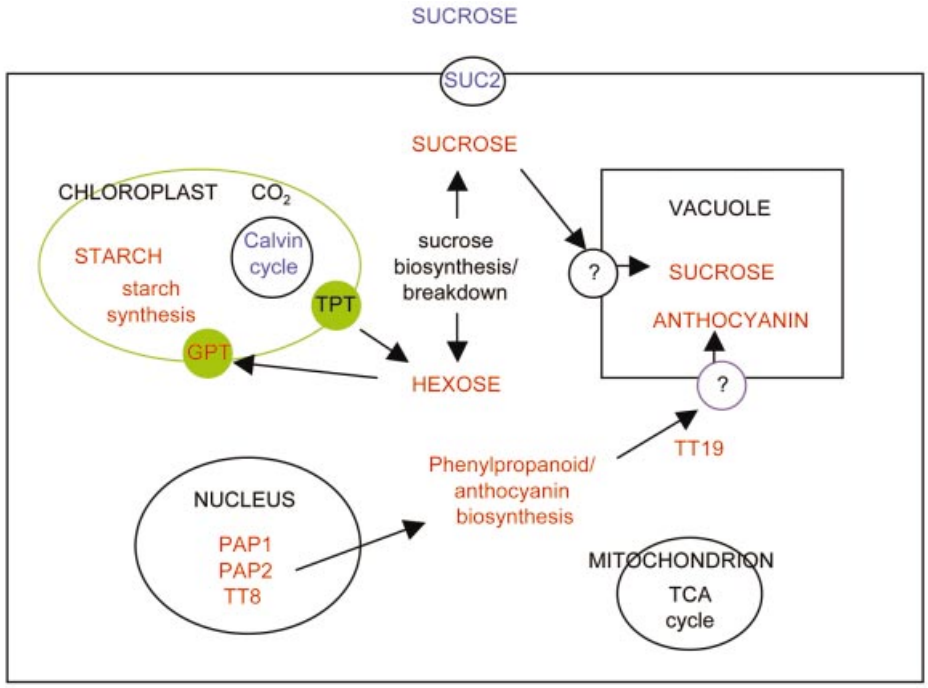

Fig. 3. Effect of the *pho3* mutation on the expression of genes involved in different metabolic pathways in mature leaves. Differences in expression between wild-type and *pho3* mutant plants are indicated by coloured typeface; red for increased and blue for decreased expression. Black typeface indicates no general significant change overall in expression.

et al. (2003). However, for most of these genes, transcript levels were unchanged suggesting that trehalose metabolism is not significantly altered in the *pho3* mutant. Only one putative trehalose-6-phosphate synthase/phosphatase (AtTPS5, At4g17770) transcript was increased, by 3-fold, in the *pho3* mutant and a further three were reduced by up to 80%.

Genes categorized as involved in signalling and transcription together accounted for 25% of the largest significant changes in expression, those expressed at more than 5-fold higher levels in the *pho3* mutant. Unfortunately, the present lack of knowledge of the function of most of these proteins limits the interpretation of the significance of changes in their transcript levels in the plant. The analysis presented shows new connections in the regulation of primary and secondary metabolism, exemplified by expression of the GPT for glucose-6 phosphate transport to the chloroplast and the regulation of the pathway for anthocyanin biosynthesis (Fig. 3). The role of sucrose, or other metabolites, and the components of the signalling pathways involved in this regulation can now be addressed by more focused experiments.

Perspective

Studies on the regulation of metabolism making use of microarrays have the potential to uncover unexpected connections, such as those described above, and reveal candidate components of signalling pathways. An explosion in the volume of this type of data is already starting as the results of microarray experiments come online. Interpretation of these microarray data should become easier as annotation of gene function becomes more comprehensive, but, at present, there are large gaps in our knowledge. The difficulties with data analysis are compounded since much published information has yet to be incorporated into the databases. However, as the proteins encoded by the *Arabidopsis* genome are identified and their function and activity within the cell is understood then microarray analysis is poised to make a major contribution to building a complete picture of metabolic regulation in plants.

Acknowledgements

This work was supported by the BBSRC (grant number 84/P13264) and the GarNET initiative. We thank Christine Raines for critical reading of this manuscript and Phil Davies for help with figures.

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